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## Alternative strategies for lignocellulose fermentation through lactic acid bacteria: the state of the art and perspectives

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1 **Alternative strategies for lignocellulose fermentation through lactic**  
2 **acid bacteria: state-of-the-art and perspectives**

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## Abstract

Lactic acid bacteria (LAB) have a long history in industrial processes as food starters and biocontrol agents, but also as producers of high-value compounds. Lactic acid, their main product, is among the most requested chemicals owing to its multiple applications including synthesis of biodegradable plastic polymers. Moreover, LAB are attracting candidates for production of ethanol, polyhydroalkanoates, sweeteners, exopolysaccharides, etc.. LAB generally have complex nutritional requirements. Moreover, they cannot directly ferment inexpensive feedstocks such as lignocellulose. This significantly increases the cost of LAB fermentation and hinders its application to produce high-volume low-cost chemicals. Different strategies have been explored to extend LAB fermentation to lignocellulosic biomass. Fermentation of lignocellulose hydrolysates by LAB has most often been reported and is the most mature technology. However, current economic constraints of this strategy have driven research for other alternative approaches. Co-cultivation of LAB with native cellulolytic microorganisms may allow to reduce the high cost of exogenous-cellulase supplementation. Special attention will be given here to construction of recombinant cellulolytic LAB by metabolic engineering which may generate strains able to directly ferment plant biomass. The present review will illustrate the state-of-the-art of these strategies and perspectives towards their application to industrial 2<sup>nd</sup> generation biorefinery processes.

**Key words:** Lactobacillus, lactococcus, cellulase, recombinant cellulolytic strategy, metabolic engineering, cellulosome

## Introduction

Lactic acid bacteria (LAB) have extensive industrial application, mainly in food fermentation and as probiotics (Mazzoli *et al.* 2014). Relevant industrial processes involving LAB also include fermentative production of lactic acid (LA). LA is among the most requested chemicals because of its several applications in food (e.g. acidifier and flavour-enhancing agent), cosmetic (emulsifying and moisturizing agent) and pharmaceutical (intermediate) industries and as building block for the synthesis of biodegradable plastic polymers (e.g. polylactides, PLAs) (Abdel-Rahman *et al.* 2013). It has been estimated that about 90% of the worldwide LA is produced through LAB fermentation (Sauer *et al.* 2008). LA can be produced also by chemical synthesis, but this gives rise to a racemic mixture of D- and L-LA which is not suitable for PLA production (Abdel-Rahman *et al.*, 2016). Furthermore, D-LA can cause metabolic problems to humans and therefore cannot be used in the food, drink, and pharmaceutical industries (Jem *et al.* 2010). Depending on specific LAB strain genome, i.e. the presence of gene(s) encoding D- or L- lactate dehydrogenase and/or racemase, D- or L-LA or their mixtures can be produced. In addition, LAB have been considered as candidates for production of other high-value compounds such as ethanol, polyhydroalkanoates, polyols, and exopolysaccharides (Mazzoli *et al.* 2014). However, most LAB are auxotrophic for several amino acids, nucleotides and vitamins (that should be supplemented to their growth media). Furthermore, LAB, with few exceptions, cannot ferment abundant inexpensive biomass, such as starchy or lignocellulosic feedstocks. These are significant limits for LAB to be applied to economically viable biorefinery processes, especially those aimed at high-volume low-value molecules (e.g. ethanol). Nowadays, most LA is produced by bioconversion of dedicated crops (mainly corn) by industries such as Corbion-Purac (The Netherlands), Galactic (Belgium), NatureWorks LLC-Cargill (USA) (Abdel-Rahman *et al.* 2013; de Oliveira *et al.* 2018). As the global demand for LA is rapidly increasing (16.2 % annual growth) (de Oliveira *et al.* 2018), such a process represents a threat for these food crops. Development of fermentation processes based on 2<sup>nd</sup> generation (i.e. lignocellulosic) feedstocks appears as a priority for extensive application of LAB in biorefinery.

So far, no native cellulolytic and/or hemicellulolytic LAB has been identified. However, a number of LAB strains have been isolated from “plant environments”, e.g. from fermented vegetables or the gastrointestinal tract of herbivores where plant biomass is the main carbon source. These LAB developed the ability to ferment a variety of soluble sugars derived from plant polysaccharide hydrolysis (see next section). Supplementation of cellulases in the growth medium (Adsul *et al.* 2007;° Wee and Ryu 2009; Shi *et al.* 2015; Bai *et al.* 2016; Hu *et al.* 2016; Overbeck

*et al.* 2016; Wang *et al.* 2017; Grewal and Khare 2018) or co-cultivation with cellulolytic microorganisms (Shahab *et al.* 2018) have therefore been used as efficient strategies to allow plant biomass fermentation by LAB. Alternatively, the development of recombinant LAB equipped with heterologous cellulase systems has been pursued so as to obtain strains that can directly ferment lignocellulosic feedstocks (i.e. consolidated bioprocessing, CBP) (Mazzoli *et al.*, 2014). The state-of-the-art of these strategies and future research directions towards their application in industrial processes will be described in the next sections.

## **LAB ability to ferment soluble mono-/oligo-saccharides from lignocellulosic biomass**

LAB can metabolize several monosaccharides, including both hexoses (e.g. fructose, glucose, galactose) and pentoses (e.g. xylose) (Kandler 1983), which are common components of lignocellulosic materials. Based on their metabolism, LAB are classified as homo-, hetero- and mixed acid-fermenters (Kandler 1983). In homofermentative metabolism, sugars are catabolized through the Embden-Meyerhof-Parnas pathway and converted to pyruvate which is finally reduced to LA. Heterofermentative metabolism involves sugar conversion through the phosphoketolase pathway giving rise to equimolar mixtures of LA and ethanol/or acetic acid (Kandler 1983). Finally, in mixed acid fermenters, glycolysis-derived pyruvate is metabolized through multiple pathways resulting in production of LA and ethanol and/or acetic and/or formic acid mixtures (Kandler 1983). Efficient metabolism of pentose sugars is particularly important when hemicellulose fermentation is addressed (Jordan *et al.* 2012). Some LAB such as *Lactobacillus (Lb.) pentosus*, *Lb. brevis*, *Lb. plantarum* and *Leuconostoc (Leu.) lactis* can metabolize both arabinose and xylose through heterofermentative metabolism (Fig. 1) (Tanaka *et al.* 2002; Okano *et al.* 2009a). An additional xylose fermentation pathway featuring higher LA production yields was identified in *Lactococcus (Lc.) lactis* IO-1 (Tanaka *et al.* 2002). In this strain, at high xylose concentration, xylose catabolism is shifted from the phosphoketolase pathway to the pentose-phosphate pathway which catalyzes its homo-lactic conversion (Fig. 1) (Tanaka *et al.* 2002).

Efficient metabolism of oligosaccharides derived from partial hydrolysis of cellulose/hemicellulose is essential for optimal fermentation of these polysaccharides (Galazka *et al.* 2010; Lane *et al.* 2015). In native cellulolytic microorganisms, a significant part of these oligosaccharides are likely not saccharified in the extracellular environment (Desvaux 2006). Instead, they are transported through specific proteins into the cytoplasm where they are further

metabolized through either hydrolytic or phosphorolytic mechanism (Desvaux 2006). Notoriously, cellodextrin transport and intracellular metabolism have been engineered in important candidates for 2<sup>nd</sup> generation biorefinery such as *Saccharomyces cerevisiae* and *Yarrowia lipolytica* (Galazka *et al.* 2010; Lane *et al.* 2015). Advantageously, an increasing number of natural LAB have been shown to metabolize cellobiose and other short cellodextrins or short oligosaccharides derived from hemicellulose (e.g. xylan,  $\beta$ -glucan) hydrolysis (Ohara *et al.* 2006; Adsul *et al.* 2007a; Kowalczyk *et al.* 2008; Okano *et al.* 2010b; Lawley *et al.* 2013). Recently, *Lc. lactis* IL1403, i.e. one of the very reference LAB strains, has shown the natural ability to ferment up to cellotetraose/cellopentaose (Gandini *et al.* 2017). This study has indicated that this strain is equipped with membrane transporters for short cellodextrins, although they have not been identified yet. The genome of this strain is rich in genes encoding putative  $\beta$ -glucosidases/6-P- $\beta$ -glucosidases, while no gene coding for cellodextrin phosphorylase is present (Bolotin *et al.* 2001). As regards the metabolism of partial hydrolysis products of hemicellulose, it is worth reminding the identification of three LAB strains, i.e. *Lc. lactis* IO-1, *Leu. lactis* SHO-47 and *Leu. lactis* SHO-54, that can ferment xylooligosaccharides with degrees of polymerization up to six (Ohara *et al.* 2006). Here again, it was demonstrated that these xylooligosaccharides are hydrolyzed by intracellular xylosidases, while transporters for their uptake were not identified (Ohara *et al.* 2006). Although rare, the presence of genes encoding enzymes involved in depolymerization of xylooligosaccharides, and/or arabinoxylans and/or arabinans (i.e.  $\beta$ -xylosidases and arabinofuranosidases) has been detected in different strains of *Lactobacillus* spp., *Pediococcus* spp., *Leuconostoc/Weissella* branch, and *Enterococcus* spp. (Michlmayr *et al.* 2013). Recently, *Lb. ruminis*, an inhabitant of human bowels and bovine rumens, has been shown to ferment tetrasaccharides derived from barley  $\beta$ -glucan (Lawley *et al.* 2013).

Since fermented vegetables and other environments rich in plant biomass are habitats in which LAB can be commonly found, it is likely that future analyses will identify further LAB strains equipped with basic biochemical systems for metabolizing sugars derived from plant material.

## **Alternative strategies for lignocellulose fermentation through LAB**

### ***Fermentation of pre-treated lignocellulosic biomass by natural LAB***

132 Since natural LAB cannot directly hydrolyze and ferment polysaccharides present in  
133 lignocellulose physical and/or chemical and/or enzymatic pre-treatment(s) of biomass are  
134 necessary. Several examples of fermentation of different pre-treated/hydrolyzed lignocellulosic  
135 feedstocks by LAB have been reported that include de-oiled algal biomass (Overbeck *et al.* 2016),  
136 barley bran (Moldes *et al.* 2006), corncob (Guo *et al.* 2010; Bai *et al.* 2016), corn stover (Hu *et al.*  
137 2016; Wang *et al.* 2017), de-oiled cottonseed cake (Grewal and Khare 2018), oak wood chip (Wee  
138 and Ryu 2009), paper mill sludge (Marques *et al.* 2008; Shi *et al.* 2015), sugarcane bagasse (Adsul  
139 *et al.* 2007; Laopaiboon *et al.* 2010), trimming vine shoots (Bustos *et al.* 2005; Moldes *et al.* 2006),  
140 wheat bran (Naveena *et al.* 2005; Li *et al.* 2010), wheat straw (Grewal and Khare 2018) (Table 1).

141 Two main technical challenges are specifically associated with this fermentation strategy: i)  
142 generation of inhibitory compounds by physico-chemical pre-treatment; ii) inefficient  
143 saccharification of biomass (for an extensive overview Abdel-Rahman *et al.* 2016). Most physico-  
144 chemical methods generate inhibitory by-products such as phenolic and furan compounds (e.g.  
145 furfural and 5-hydroxymethylfurfural), organic acids (e.g. acetic, formic, and levulinic acid) and  
146 alcohols (Zhang *et al.* 2016a). The latter may negatively interfere with the activity of  
147 cellulolytic/hemicellulolytic enzymes and/or the metabolism of fermenting strains (Abdel-Rahman  
148 *et al.* 2016). Furthermore, enzymatic hydrolysis of plant polysaccharides frequently suffers from  
149 inhibition by end-product (e.g. glucose, cellobiose) accumulation (Abdel-Rahman *et al.* 2016). For  
150 this reason, separate hydrolysis and fermentation (SHF) approach can be advantageously replaced  
151 by simultaneous saccharification and fermentation (SSF) strategy. The latter minimizes end-product  
152 inhibition of hydrolases through rapid consumption of soluble sugars by fermenting  
153 microorganisms (Lynd *et al.* 2002). Furthermore, lignocellulose fermentation suffers from the  
154 complex nature of this biomass, consisting of different polysaccharides (mainly cellulose,  
155 hemicelluloses and pectin) (Lynd *et al.* 2002). Lignocellulose hydrolysis generates sugar mixtures  
156 which may undergo inefficient fermentation caused by heterofermentation of pentoses (see previous  
157 section) and/or carbon catabolite repression (Jojima *et al.* 2010). The latter refers to inhibition of  
158 pentose metabolism by the presence of glucose leading to non-simultaneous fermentation of sugar  
159 mixtures that often leaves most sugar unutilized (Abdel-Rahman *et al.* 2016). A wide variety of  
160 solutions can be employed to overcome these limitation(s) (Abdel-Rahman *et al.* 2011; 2016).  
161 Strategies to reduce the concentration of inhibitory compounds include the choice of alternative  
162 milder physico-chemical pre-treatments (e.g. acid or alkaline treatment, steam explosion, ionic  
163 liquids) (Abdel-Rahman *et al.* 2011) and methods (e.g. chemical additives such as ion exchange  
164 resins, bioabatement) for detoxifying pre-treated biomass (Laopaiboon *et al.* 2010; Jönsson and  
165 Martín 2016). Alternatively, the use of enzymes and LAB strains with higher tolerance to these

166 compounds (either natural or obtained through evolutionary or rational engineering) is a valuable  
167 option (Abdel-Rahman *et al.* 2016). Cellulase mixtures with different composition and different  
168 configurations of the fermentative process (e.g. SHF and SSF) can be used to optimize specific  
169 biomass hydrolysis (Abdel-Rahman *et al.* 2016). Finally, several LAB strains showing highly  
170 efficient metabolism of pentoses are known. Homolactic fermentation of xylose has been observed  
171 in *Lc. lactis* IO-1 (Tanaka *et al.* 2002) or *E. faecium* QU 50 (Abdel-Rahman *et al.* 2015). Several  
172 LAB showing relaxed carbon catabolite repression have been reported. For instance, different *Lb.*  
173 *brevis* strains were able to simultaneously utilize xylose and glucose derived from hydrolysis of a  
174 variety of lignocellulosic feedstocks (Guo *et al.* 2010; Grewal and Khare 2018), while *E. faecalis*  
175 RKY1 co-metabolized mixtures of sucrose, glucose, and/or fructose to LA with high yield (Reddy  
176 *et al.* 2015) and *E. faecium* QU 50 homofermentatively utilized glucose/xylose mixtures (Abdel-  
177 Rahman *et al.* 2015). Additionally, metabolic engineering strategies have been used to develop  
178 strains with improved pentose catabolism, as described in the following sections.

179         Actually, some studies demonstrate that very efficient bioconversion of lignocellulosic  
180 biomass into nearly optically pure LA through LAB fermentation (with LA yields close to the  
181 theoretical maximum) can be obtained by selecting optimal combination of pre-treatment, process  
182 configuration and microbial strain suitable for a specific substrate (Table 1). However, both  
183 physico-chemical and enzymatic treatments utilized in these studies have significant costs which  
184 represent relevant economic barriers at the industrial scale (Okano *et al.* 2010a). Despite extensive  
185 research efforts for reducing the cost of production of cellulases, no significant decrease has been  
186 observed since the 1990s (Olson *et al.* 2012). A recent study has estimated the cost of at-site  
187 production of cellulases as \$10/kg protein (the cost of commercial cellulases is higher) (Klein-  
188 Marcuschamer *et al.* 2012). Based on calculations used by Lynd *et al.* (2017), it can be estimated  
189 that the cost of added cellulases per kg LA produced through lignocellulose fermentation cannot be  
190 lower than 0.31 \$. It is worth noting that the cost of fermentative production of LA should be at or  
191 below 0.8 \$ /kg for PLA to be economically competitive with fossil fuel-based plastics (Okano *et*  
192 *al.* 2010a). Such an economic target is therefore very challenging through processes such as those  
193 described in this section, where the cost of physico-chemical and enzymatic pre-treatment risks to  
194 be significantly too high. Some techno-economic analyses of LA production from renewable  
195 biomass have been recently summarized by de Oliveira *et al.* (2018). Costs may widely vary  
196 depending on the process configuration (e.g. type of feedstocks, method for biomass pre-treatment,  
197 LA purification process). In most cases the minimum LA sell price was higher than 0.8 \$ /kg (i.e.  
198 between 0.83 and 5 \$ /kg). However, a recent study reported a minimum sell price of 0.56 \$ /kg for  
199 LA produced through fermentation of pre-treated (i.e. dilute acid plus enzymatic hydrolysis) corn



200 stover (Liu et al. 2015). Interestingly, cellulase cost was reported as the highest in the entire process  
201 (Liu et al. 2015). Research for alternative strategies for lignocellulose fermentation with lower  
202 dependence on biomass pre-treatment(s) is therefore highly recommended. Significant attention has  
203 been dedicated to the so-called CBP, i.e. single-pot fermentation of lignocellulosic biomass  
204 (Mazzoli 2012). This process configuration differs from SHF and SSF especially in that it does not  
205 involve a dedicated process step for cellulase production (Lynd *et al.* 2005). This could be obtained  
206 through cellulolytic microorganisms-LAB consortia or by engineering cellulolytic ability in LAB. It  
207 has been calculated that CBP could lower cost of biological conversion of lignocellulose by about  
208 78 % (Lynd *et al.* 2005).

209

#### 210 ***Fermentation of lignocellulosic biomass by cellulolytic microorganisms-LAB consortia***

211 Co-cultivation of LAB with native cellulolytic microorganisms could interestingly replace  
212 saccharification of lignocellulosic biomass by exogenously supplemented cellulases. Utilization of  
213 microbial consortia including cellulolytic strains and high-value compound producing microbes has  
214 been successfully applied to convert cellulosic feedstocks to a variety of products such as ethanol or  
215 butanol (Zuroff *et al.* 2013; Brethauer and Studer 2014; Wen *et al.* 2014). To date, a single  
216 application of this strategy to production of LA by LAB fermentation has been reported (Shahab *et*  
217 *al.* 2018). In this study, a stable consortium between the cellulolytic fungus *Trichoderma reesei* and  
218 *Lb. pentosus* based on mutual benefits was developed (Fig. 2). *Lb. pentosus* efficiently consumes  
219 cellobiose thus avoiding inhibition of *T. reesei* cellulase activity. On the other hand, a by-product of  
220 sugar fermentation by *Lb. pentosus*, i.e. acetic acid, can serve as carbon source for *T. reesei* (Shahab  
221 *et al.* 2018). Fermentation of whole-slurry pre-treated beech wood by this consortium led to  
222 production of 19.8 g/L of LA, with an estimated yield of 85.2% of the theoretical maximum,  
223 through CBP (Shahab *et al.* 2018). This study demonstrates that this approach, that mimics  
224 microbial syntrophic communities involved in natural decay of plant material, deserves further  
225 investigation. In parallel, difficulties related to design and maintain stable artificial microbial  
226 communities represent main challenges of this strategy (Johns *et al.* 2016).

227

#### 228 ***Construction of recombinant cellulolytic/hemicellulolytic LAB through metabolic engineering:*** 229 ***state of the art and future directions.***

Forefront research in development of 2<sup>nd</sup> generation biorefinery includes endowing microorganisms that produce high-value chemicals with the ability to directly ferment lignocellulose without prior physico-chemical and/or enzymatic pre-treatment through recombinant techniques (Mazzoli *et al.* 2012). The number of examples of recombinant cellulolytic strategies (RCS) addressed to LAB is growing (Mazzoli *et al.* 2014; Gandini *et al.* 2017; Stern *et al.* 2018). Natural ability to grow on lignocellulose relies on multiple-enzyme systems that mainly consist of glycosyl hydrolases and polysaccharide lyases (Lynd *et al.* 2002). Most studies have been addressed to two main paradigms for cellulose depolymerization, the non-complexed enzyme model of aerobic fungi and bacteria and the cellulosome complexes of anaerobic microorganisms (Lynd *et al.* 2002). The latter are based on scaffolding proteins (i.e. scaffoldins) that generally provide multiple functions, i.e. the ability to bind enzyme subunits (thus organizing the enzyme complex architecture), polysaccharides and cell surface through specific protein domains (Mazzoli *et al.* 2012). RCS aim at mimicking nature by engineering minimal cellulolytic systems (Mazzoli 2012) (Fig. 3). Traditionally, a minimal non-complexed system able to act efficiently on cellulosic substrates consists of an exoglucanase, an endoglucanase and a  $\beta$ -glucosidase (Lynd *et al.* 2002) (Fig. 3A, B). A mini-scaffoldin is also required in the case of mini- or designer-cellulosomes (Fig. 3C). However, in most studies aimed at LAB engineering with heterologous cellulases reported so far, a single cellulase was introduced (for an extensive review, Mazzoli *et al.* 2014) (Table 2). This modification may enable metabolization of short cellodextrins or partial hydrolysis of cellulose/hemicellulose but is insufficient for these recombinant strains to efficiently grow on and ferment complex lignocellulosic substrates (Mazzoli *et al.* 2014) (Table 2). Actually, most of these recombinant strains were aimed at being used as inoculants for silage fermentation (i.e. for improving silage acidification and/or digestibility) (Bates *et al.* 1989; Scheirlinck *et al.* 1989; Rossi *et al.* 2001; Ozkose *et al.* 2009) rather than as biocatalysts in biorefinery processes. More recently, construction of cellulolytic LAB for industrial production of LA has been considered. Among the most performant strains, *Lb. plantarum* engineered with Cel8A endoglucanase from *C. thermocellum* was able to grow on cellooligosaccharides long up to 5-6 glucose residues (Okano *et al.* 2010b). Several studies have reported that expression of heterologous cellulases may be toxic (Mingardon *et al.* 2011; Morais *et al.* 2014). Hence, expression of multiple cellulases is extremely challenging. The development of artificial syntrophic consortia (consisting of recombinant strains that biosynthesize single different cellulase-system components) has been used to circumvent this bottleneck (Morais *et al.* 2013; 2014; Stern *et al.* 2018). Morais and co-workers (2013) have shown the potential of simple consortia of recombinant *Lb. plantarum* strains secreting cellulase-xylanase mixtures for biomass (i.e. wheat straw) bioconversion. The same research group has significantly

improved its hemi/cellulolytic LAB consortium over time by including strains that biosynthesize different: i) surface-anchored mini-scaffoldins (each able to bind up to 4 enzymatic subunits); ii) adaptor mini-scaffoldins (each able to bind up to 2 enzymatic subunits) and; iii) endoglucanases and xylanases (Morais *et al.* 2014; Stern *et al.* 2018) (Fig. 3C). Synthetic *Lb. plantarum* consortia that display mini-cellulosomes incorporating up to six enzymatic subunits could be developed, which is a remarkable result (Stern *et al.* 2018). Although these enzyme complexes showed improved hydrolysis of wheat straw, they were unable to support growth of *Lb. plantarum* on wheat straw as the sole carbon source. This result is likely related to the amount and/or type of sugars released by the specific designer cellulosomes which seems insufficient/unsuitable for *Lb. plantarum* growth (Stern *et al.* 2018). Additionally, it has to be reminded that management of these consortia at the industrial scale may not be trivial. Recently, a cellulase system consisting of a  $\beta$ -glucosidase and an endoglucanase has been engineered in a single *Lc. lactis* strain through construction of an artificial operon (Gandini *et al.* 2017). This strain could directly convert cellooligosaccharides up to at least cellooctaose to L-LA with high yield. However, the basal expression triggered by the used promoter (P32) did not show to be very high, and further improvement of this strain towards application in biorefinery will be required, e.g. through increased cellulase expression (Gandini *et al.* 2017).

Attempts to improve hemicellulose metabolism in LAB include few examples of expression of heterologous xylanases (Raha *et al.* 2006; Morais *et al.* 2013; Gandini *et al.* 2017) (Table 3). Morais *et al.* (2013) demonstrated that xylanase-expressing *Lb. plantarum* improved cellulose accessibility. Most other metabolic engineering studies have concerned the improvement of pentose conversion into LA through disruption of the phosphoketolase pathway and introduction or enhancement of the pentose phosphate pathway (Okano *et al.* 2009 a; b; Shinkawa *et al.* 2011; Qiu *et al.* 2017) (Table 2). These studies obtained impressive results since engineered strains were able of nearly homolactic fermentation of xylose and/or arabinose (Table 3). Additionally, some engineered strains showed the ability to co-ferment glucose/xylose mixtures without carbon catabolite repression (Yoshida *et al.* 2011; Zhang *et al.* 2016b).

Although the number of RCS targeted to LAB engineering is growing, research progress on these organisms is still far behind that obtained in other microbial models, such as *S. cerevisiae*. All abovementioned examples suffer from multiple limits which hamper application of such recombinant LAB to industrial fermentation of real cellulosic substrates. In most cases, inducible promoters have been used to control the transcription of heterologous cellulases (Table 2). Inducible promoters have been preferred so as to delay cellulase expression in the late exponential phase, thus avoiding major growth inhibition by cellulase expression. However, utilization of inducible

promoters is not cost-sustainable at the industrial scale, since large amounts of expensive inducer should be employed. A further problem may be represented by the limited amount of cellulases which are secreted by the recombinant cellulolytic LAB obtained so far (Table 2) (Mazzoli *et al.* 2014). As a basis for comparison, cellulase activity of native cellulosome-producing *Clostridium thermocellum* on cellulosic substrates can range between 100 and 1000 U/L (Krauss *et al.* 2012; You *et al.* 2012). In many state-of-the-art recombinant cellulolytic LAB, measured cellulolytic activities are around or under the lower side of this range (Table 2) and are strongly dependent on specific cellulase (Stern *et al.* 2018). Although available genetic tools for LAB are relatively abundant, those enabling strong constitutive expression of proteins have long been restricted to few choices, such as the lactococcal P32 and P45 promoters (Table 2). Luckily, new constitutive promoters with different strengths are being discovered for both Lactococci (Zhu *et al.* 2015) and Lactobacilli (Duong *et al.* 2011; Tauer *et al.*; 2014). Alternatively, generation of libraries of synthetic constitutive promoters displaying a wide range of strength (Jensen and Hammer 1998; Rud *et al.* 2006) seems a potent tool to mimicking native cellulase systems in which the highest synergism is obtained for non-equimolar expression of different enzymes (Mazzoli *et al.* 2012). Additional tools to increase cellulase/hemicellulose expression in LAB include improvement of mRNA stability (Narita *et al.* 2006; Okano *et al.* 2010) or increase of translation efficiency through design of synthetic genes with optimized codon usage (Johnston *et al.* 2014; Dong *et al.* 2015; Li *et al.* 2016). The most challenging factor in heterologous expression of cellulases consists in finding efficient secretion strategy (Mazzoli *et al.* 2012). Saturation of transmembrane transport mechanisms of the host and accumulation of misfolded or aggregated proteins is the most probable factor causing toxicity of heterologous cellulases (Illmen *et al.* 2011; Morais *et al.* 2014). Mechanisms of cellulase secretion in native cellulolytic microorganisms are almost completely unknown. Based on analysis of signal peptide sequence, a recent study postulated that only about 6% of the known cellulases is secreted through established mechanisms (e.g. the Sec or Tat pathway) (Yan and Wu 2014). In this scenario, studies on heterologous cellulase expression have often been based on trial-and-error approach so as to find enzymes compatible with the host (Illmen *et al.* 2011; Mingardon *et al.* 2011). Luckily, mechanisms of protein secretion in cellulolytic clostridia and LAB have shown some similarities since a number of components of cellulase systems of clostridia, with their original signal peptide, could be efficiently secreted by *Lb. plantarum* or *Lc. lactis* (Okano *et al.* 2010b; Wieczorek and Martin 2010; Morais *et al.* 2013; Gandini *et al.* 2017). Alternatively, original signal peptides of cellulases can be replaced with sequences (i.e. signal peptides, propeptides) promoting efficient protein secretion in the host of interest (Dong *et al.* 2015; Lim *et al.* 2017). Typically, the native (or engineered) signal peptide of

331 Usp45, the main secreted protein of *Lc. lactis*, has been used for promoting the secretion of  
332 heterologous proteins in *Lc. lactis* (Morello *et al.* 2008; Ng and Sarkar 2013), including cellulase  
333 system components from different microorganisms (Wieczoreck and Martin 2010; Wang *et al.*  
334 2014; Liu *et al.* 2017), while Lp3050 or Lp2588 leader peptides have been used to enable secretion  
335 of cellulosomal components in *Lb. plantarum* (Stern *et al.* 2018). All these tools can significantly  
336 help development of RCS of LAB, however, they cannot guarantee their success that currently still  
337 mainly depends on specific protein/host combination. Signal peptides and propeptides likely play  
338 additional roles in protein translocation, maturation and folding which need better understanding  
339 (Harwood and Cranenburgh 2008; Mazzoli *et al.* 2012; Yan and Wu 2014). Furthermore, unusual  
340 mechanisms of protein folding have been speculated for some cellulases which may require  
341 assistance by specific chaperon(s) (Mingardon *et al.* 2011). For instance, co-expression of  
342 chaperon-like *B. subtilis* PrsA protein was able to improve secretion yield of heterologous amylase  
343 and penicillinase in *Lc. lactis* (Lindholm *et al.* 2006). Increase of secretion yield of heterologous  
344 cellulases may also be obtained by inactivation of housekeeping protease(s), as demonstrated by *Lc.*  
345 *lactis* mutants defective in the unique exported housekeeping protease HtrA (Wieczoreck and  
346 Martin 2010). Co-expression of protease inhibitors found as integral components of some clostridial  
347 cellulosomes (Meguro *et al.* 2011; Xu *et al.* 2014) could be an alternative strategy worth being  
348 tested.

349         Apart from improving the amount of cellulolytic enzymes, future directions in construction  
350 of recombinant cellulolytic LAB should focus on improving synergism of designer cellulase  
351 systems. Expression of multiple enzymes with highly complementary activities, preferably in a  
352 single strain, is essential for developing strains aimed at CBP of complex substrates. Apart from  
353 “traditional” cellulase activities (i.e. exoglucanases, endoglucanases and  $\beta$ -glucosidases, Fig. 3),  
354 attention should be addressed also to recently discovered cellulose-active proteins, such as  
355 microbial expansins (Chen *et al.* 2016) and lytic polysaccharide monooxygenases (LPMOs) (Liang  
356 *et al.* 2014). The latter could significantly improve depolymerization of most recalcitrant  
357 polysaccharides, such as crystalline cellulose. Gene integration into the LAB chromosome seems  
358 the most suitable strategy to construct genetically stable strains that co-express multiple cellulases.  
359 An extensive literature on integrative gene expression systems in LAB is available, although it is  
360 mainly focused on lactobacilli and *Lc. lactis* (for extensive reviews refer to Gaspar *et al.* 2013 and  
361 Bravo and Landete 2017). Molecular tools for unlabelled (i.e. without insertion of antibiotic  
362 resistance markers) gene integration in the LAB genomic DNA include homologous recombination  
363 (e.g. pORI, pSEUDO and Cre-lox systems) or single-stranded DNA recombineering (Gaspar *et al.*  
364 2013; Bravo and Landete 2017). Some of them have already been used to improve pentose

365 metabolism in different LAB strains (Table 3), but more extensive application to expression of  
366 heterologous hemi/cellulase systems seems necessary for significant progress of RCS in LAB.

367 Surface-display of proteins is also a valuable tool for increasing cellulase activity in LAB.  
368 This strategy mimics some of the most efficient cellulose depolymerization systems found in nature  
369 (e.g. cellulosome), where cellulase activity is improved by rapid metabolism of cellulose hydrolysis  
370 products promoted by enzyme-cell proximity (Wieczoreck and Martin 2010). So far, studies in this  
371 direction have been reported by two research groups only, i.e. that of Prof. Martin in Canada  
372 (Wieczoreck and Martin 2010; 2012) and that coordinated by Profs. Mizrahi and Bayer in Israel  
373 (Morais *et al.* 2014; Stern *et al.* 2018). While direct binding of glycosyl hydrolases to the LAB  
374 surface may cause allosteric hindrance and diminish enzyme/protein activity (Morais *et al.* 2014;  
375 Stern *et al.* 2018), surface display of mini-cellulosomes seems a good compromise for improving  
376 enzyme-cell synergism without major negative effects on cellulase flexibility and activity (Morais  
377 *et al.* 2014). Furthermore, cellulosomes were shown to improve enzyme stability (Stern *et al.* 2018).  
378 Multiple tools for protein surface-display in LAB through covalent (i.e. sortase-mediated) and non-  
379 covalent (e.g. LysM domains) binding have been reported (Okano *et al.* 2008; Wieczoreck and  
380 Martin 2010; Morais *et al.* 2014; Zadavec *et al.* 2015) and can be used to further developing these  
381 strategies.

382

## 383 **Conclusion**

384 LAB have long been used for industrial purposes and show good characteristics for future  
385 application also to 2<sup>nd</sup> generation biorefinery. Generally, they can metabolize several  
386 monosaccharides which are components of plant biomass, including both hexoses and pentoses.  
387 Some of them can directly ferment short cello- or xylo-oligosaccharides or co-ferment hexoses and  
388 pentoses without carbon catabolite repression. Successful examples of LAB fermentation of  
389 hydrolyzed lignocellulosic feedstocks (e.g. algal cake, corncob, corn stover, paper mill sludge,  
390 sugarcane bagasse, trimming vine shoots, wheat straw) have been reported. However, the high cost  
391 of physico-chemical pre-treatment and of the high amounts of commercial cellulases needed for  
392 biomass saccharification are major barriers towards industrial application of these technologies.  
393 Waiting for development of cheaper pre-treatments or cellulase-production processes, research for  
394 alternative lignocellulose-LAB fermentation strategies is in progress. Synthetic consortia of  
395 cellulolytic microorganisms and LAB may eliminate the need for exogenous cellulases through an  
396 approach that mimics natural microbial communities involved in plant biomass decay. The main

challenge here is represented by maintaining such stable consortia at the industrial scale, but the studies reported in the literature encourage pursuing research along this, so far scarcely investigated, path. Recombinant strategies aim at engineering LAB with heterologous cellulase systems able to directly ferment lignocellulose without any external help. This strategy promises huge process cost reduction, but is highly challenging. Despite the relatively high number of gene tools available for LAB, RCS suffer from intrinsic toxicity of many heterologous cellulases and from lignocellulose recalcitrance requiring expression of multiple synergistic enzyme activities. Recombinant LAB obtained so far cannot grow on cellodextrins longer than 8-9 glucose units and intense research efforts will be needed towards direct fermentation of lignocellulosic feedstocks.

In conclusion, interesting progress towards LAB application in 2<sup>nd</sup> generation biorefinery has been made. Since finding alternative energies is currently a global priority, it can be hoped that new economic resources will help further developments in this research area. In this perspective, each alternative strategy presented in this review represents a promising opportunity.

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Table 1. Examples of LAB fermentation of pre-treated lignocellulosic biomass. Strategies for physico-chemical and/or enzymatic pretreatment of biomass are summarized. n.r., not reported; SHF, separate hydrolysis and fermentation; SSF simultaneous saccharification and fermentation. In this table, the term SSF has been employed for processes featuring simultaneous saccharification and fermentation of all the soluble sugars derived from biomass hydrolysis that, depending of the biomass composition, may be hexoses or pentoses or mixtures (i.e. co-fermentation).

| Biomass                             | Physico-chemical treatment(s)  | Enzymatic treatment  | Microorganisms                            | Fermentation mode | LA enantiomer         | LA (g/L) | Yield $Y_{P/S}$ (g/g) | Productivity (g/L/h) | References                    |
|-------------------------------------|--|--|---|-------------------|-----------------------|----------|-----------------------|----------------------|-------------------------------|
| Algal cake (de-oiled algal biomass) | -  | Porcine pepsin (37°C, 3 h) plus $\alpha$ -amylase (37°C, 16 h) plus endo-1,4- $\beta$ -D-glucanase (50°C, 24h) from <i>Aspergillus niger</i> | <i>Lb. casei</i> 12A                      | SHF Batch         | L- (and traces of D-) | 11.17    | -                     | -                    | Overbeck <i>et al.</i> , 2016 |
| Barley bran                         | Biomass was dried, milled and hydrolyzed with 3% H <sub>2</sub> SO <sub>4</sub> (130°C, 15 min)  | -  | <i>Lb. pentosus</i> CECT-4023T            | SHF Batch         | n.r.                  | 33       | 0.57 <sup>a</sup>     | 0.60                 | Moldes <i>et al.</i> , 2006   |
| Birch wood xylan                    | -  | Xylanase (1.25 g/L) (60°C, 20 min)   | <i>Leu. lactis</i> SHO-47                 | SHF Batch         | D-                    | 2.3      | -                     | -                    | Ohara <i>et al.</i> , 2006    |
| Corn cob                            | Biomass was dried, milled and hydrolyzed with 2% H <sub>2</sub> SO <sub>4</sub> (130°C, 15 min)  | -  | <i>Lb. pentosus</i> CECT-4023T            | SHF Batch         | n.r.                  | 26       | 0.53 <sup>a</sup>     | 0.34                 | Moldes <i>et al.</i> , 2006   |
| Corn cob                            | Biomass was mashed and hydrolyzed with 0.1% H <sub>2</sub> SO <sub>4</sub> (80°C, 1 h) and 0.8% H <sub>2</sub> SO <sub>4</sub> (110°C, 2h) | -  | <i>Lb. brevis</i> S3F4                    | SHF Batch         | n.r.                  | 39.1     | 0.69 <sup>a</sup>     | 0.81                 | Guo <i>et al.</i> , 2010      |
| Corn cob residue                    | -  | Commercial cellulase mixture (15 FPU/g biomass)  | <i>Sporolactobacillus inulinus</i> YBS1-5 | SHF Fed-Batch     | D-                    | 107.2    | 0.85 <sup>b</sup>     | 1.19                 | Bai <i>et al.</i> , 2016      |
| Corn stover                         | Biomass was mashed   | -  | <i>Lb. brevis</i> S3F4                    | SHF Batch         | n.r.                  | 18.2     | 0.74 <sup>a</sup>     | 0.76                 | Guo <i>et al.</i> ,           |

|                                       |   |  |  |   |      |       |                   |      |                              |
|---------------------------------------|---|--|--|---|------|-------|-------------------|------|------------------------------|
|                                       | and hydrolyzed with 2% H <sub>2</sub> SO <sub>4</sub> at a 10% (w/v) (121°C, 1h)  |  |  |   |      |       |                   |      | 2010                         |
| Corn stover                           | Biomass was dried, sieved and treated with 5% NaOH (75°C, 3 h)  | Commercial cellulase, $\beta$ -glucosidase, and xylanase mixture (30 FPU/g biomass)                  | <i>Lb. pentosus</i> FL0421   | SSF Fed-batch   | n.r. | 92.30 | 0.66 <sup>c</sup> | 1.92 | Hu <i>et al.</i> , 2016      |
| Corn stover                           | Biomass was crushed, sieved, dried and treated with 1.5% solid acid (120°C, 80 min)   | Commercial cellulase mixture (30 FPU/g biomass)  | <i>Lactobacillus delbrueckii delbrueckii</i> sp. <i>bulgaricus</i> CICC21101 | SSF Batch   | D-   | 18    | -                 |      | Wang <i>et al.</i> 2017      |
| Deoiled cottonseed cake               | Biomass was ground, sieved and mixed with ionic liquid (120°C, 2 h)   | Immobilized cellulases (25 FPU/g biomass) from <i>Trichoderma reesei</i>                             | <i>Lactobacillus brevis</i> MTCC 4460  | SSF Batch   | n.r. | -     | 0.22 <sup>c</sup> | -    | Grewal and Khare, 2018       |
| Detoxified <i>Eucalyptus globulus</i> | Biomass was dried, milled, and hydrolyzed with 3% H <sub>2</sub> SO <sub>4</sub> (130°C, 1h). Hydrolysate was neutralized with CaCO <sub>3</sub> and stirred with 15% w/v of charcoal (room temperature, 1 day) | -  | <i>Lb. pentosus</i> CECT-4023T   | SHF Batch   | n.r. | 14.5  | 0.70 <sup>a</sup> | 0.28 | Moldes <i>et al.</i> , 2006  |
| Oak wood chip                         | Biomass was treated with 0.5% H <sub>2</sub> SO <sub>4</sub> (room temperature, overnight) and steam explosion (215°C, 5')  | Commercial cellulase mixture (20 IU/g) supplemented with $\beta$ -glucosidase (30 IU/g) (50°C, 48 h) | <i>Lactobacillus</i> sp. RKY2  | SHF Continuous cell recycle (dilution rate 0.16 h <sup>-1</sup> ) | n.r. | 42    | 0.95 <sup>b</sup> | 6.7  | Wee and Ryu, 2009            |
| Recycled paper sludge                 | Biomass was neutralized with 0.3 g HCl/g biomass  | -  | <i>Lb. rhamnosus</i> ATCC 7469   | SSF Batch   | n.r. | 73    | 0.97 <sup>a</sup> | 2.9  | Marques <i>et al.</i> , 2008 |
| Softwood pre-                         | Softwood particles  | Commercial cellulases  | <i>Lactobacillus</i>   | SSF Batch   | n.r. | 60    | 0.83 <sup>d</sup> | 0.62 | Shi <i>et al.</i> ,          |

|                                    |  |  |                                       |                          |      |      |                   |       |   |
|------------------------------------|--|--|---------------------------------------|--------------------------|------|------|-------------------|-------|---|
| hydrolysate plus paper mill sludge | were sieved and pre-treated with hot-water   | (15 FPU/g glucan) plus pectinases (15 mg protein/g mannan)               | <i>rhamnosus</i> ATCC-10863           |                          |      |      |                   |       | 2015                                    |
| Sugarcane bagasse                  | Biomass shreds (1–3 mm size) were pre-treated with steam and alkali  | Enzyme preparation from <i>Penicillium janthinellum</i>                  | <i>Lb. delbrueckii</i> mutant Uc-3    | SSF Batch                | L-   | 67   | 0.83 <sup>e</sup> | 0.93  | Adsul <i>et al.</i> , 2007 <sup>o</sup> |
| Sugarcane bagasse                  | Biomass was dried, milled, treated with 10% NH <sub>4</sub> OH and hydrolyzed with 0.5 % HCl (100°C, 5h). Hydrolysate was detoxified by amberlite treatment. | -  | <i>Lc. lactis</i> IO-1 JCM 7638       | SHF Batch                | n.r. | 10.9 | -                 | 0.14  | Laopaiboon <i>et al.</i> , 2010         |
| Sugarcane bagasse                  | Biomass was ground, sieved and mixed with ionic liquid (120°C, 2 h)  | Immobilized cellulases (25 FPU/g biomass) from <i>Trichoderma reesei</i> | <i>Lactobacillus brevis</i> MTCC 4460 | SSF Batch                | n.r. | -    | 0.52 <sup>c</sup> | -     | Grewal and Khare, 2018                  |
| Trimming vine shoots               | Biomass was dried, milled and hydrolyzed with 3% H <sub>2</sub> SO <sub>4</sub> (130 °C, 15 min)   | -  | <i>Lb. pentosus</i> CECT-4023T        | SHF Batch                | n.r. | 46   | 0.78 <sup>a</sup> | 0.933 | Bustos <i>et al.</i> , 2005             |
| Trimming vine shoots               | Substrate was dried, milled and hydrolyzed with 3% H <sub>2</sub> SO <sub>4</sub> (130 °C, 15 min).  | -  | <i>Lb. pentosus</i> CECT-4023T        | SHF Batch                | n.r. | 24   | 0.76 <sup>a</sup> | 0.51  | Moldes <i>et al.</i> , 2006             |
| Wheat bran                         | Biomass was pre-reduced and sterilized   | -  | <i>Lb. amylophilus</i> GV6            | Solid state fermentation | L-   | -    | 0.23 <sup>c</sup> | -     | Naveena <i>et al.</i> , 2005b           |
| Wheat bran                         | Biomass was treated with 1.5% H <sub>2</sub> SO <sub>4</sub> (ratio 1:4 w/v) (80°C, 20 h)  | -  | <i>Lb. rhamnosus</i> LA-04-1          | SHF Batch                | L-   | 75   | 0.99 <sup>b</sup> | 3.75  | Li <i>et al.</i> , 2010b                |
| Wheat straw                        | Biomass was ground, sieved and mixed with ionic liquid (120°C, 2 h)  | Immobilized cellulases (25 FPU/g biomass) from <i>Trichoderma</i>        | <i>Lactobacillus brevis</i> MTCC 4460 | SSF Batch                | n.r. | -    | 0.49 <sup>c</sup> | -     | Grewal and Khare, 2018                  |

|  |  |               |  |  |  |  |  |  |  |
|--|--|---------------|--|--|--|--|--|--|--|
|  |  | <i>reesei</i> |  |  |  |  |  |  |  |
|--|--|---------------|--|--|--|--|--|--|--|

<sup>a</sup>g of LA /g of total sugar in the hydrolysate

<sup>b</sup>g of LA / g of glucose in the hydrolysate

<sup>c</sup>g of LA / g of biomass

<sup>d</sup>g of LA / g of total hexose sugars

<sup>e</sup>g of LA / g of cellulose in the biomass

Table 2. Examples of recombinant cellulolytic strategies (RCS) on lactic acid bacteria (LAB). Recombinant LAB strains listed here were engineered with heterologous cellulase/hemicellulose systems. *Lb.*, *Lactobacillus*; *Lc.*, *Lactococcus*

| Strains  | Heterologous protein(s) expressed  | Transcriptional promoter          | Gene cloning strategy  | Heterologous protein expression/secretion level <sup>a</sup> | Improved phenotypic properties of the strain             | References               |
|--|--|-----------------------------------|------------------------|--|--|--------------------------|
| <i>Lb. gasseri</i> ATCC 33323                    | Ce8IA endoglucanase from <i>Clostridium thermocellum</i>   | inducible (lacA promoter)         | Plasmid                | 722 U/L (CMC) <sup>b</sup>                                   | Hydrolysis of CMC  | Cho et al., 2000         |
| <i>Lb. jonhsonii</i> NCK 88                      | Cel8A endoglucanase from <i>C. thermocellum</i>  | inducible (lacA promoter)         | Plasmid                | 759 U/L (CMC) <sup>b</sup>                                   | Hydrolysis of CMC  | Cho et al., 2000         |
| <i>Lb. plantarum</i> strains B41 and Lp80        | Cel8A cellulase from <i>Bacillus sp.</i> N-4   | Not indicated                     | Chromosome integration | 34.24/43.61 U/L (CMC) <sup>b</sup>                           | Increased silage acidification                           | Rossi et al., 2001       |
| <i>Lb. plantarum</i> Lp80                        | Cel8A endoglucanase from <i>C. thermocellum</i>  | Not indicated                     | Chromosome integration | ≈ 90 U/L (CMC) <sup>b</sup>                                  | Hydrolysis of CMC  | Scheirlinck et al., 1989 |
| <i>Lb. plantarum</i> NCDO 1193                   | Cel5E endoglucanase from <i>C. thermocellum</i>  | Not indicated                     | Plasmid                | 1996 U/L (CMC) <sup>b</sup>                                  | Hydrolysis of CMC  | Bates et al., 1989       |
| <i>Lb. plantarum</i> NCIMB 8826 ( $\Delta$ ldh1) | Cel8A endoglucanase from <i>C. thermocellum</i>  | Constitutive (ClpC core promoter) | Plasmid                | 6.03 U/L (barley $\beta$ -glucan) <sup>b</sup>               | Growth on cellohexaose                                   | Okano et al., 2010b      |
| <i>Lb. plantarum</i> WCFS1                       | Cel6A endoglucanase from <i>Thermobifida fusca</i>   | Inducible (sakacin P promoter)    | Plasmid                | 280 U/L (PASC) <sup>b</sup>                                  | Hydrolysis of sodium hypochlorite-pretreated wheat straw | Morais et al., 2013      |
| <i>Lb. plantarum</i> WCFS1                       | Xyn11A endoxylanase from <i>T. fusca</i>   | Inducible (sakacin P promoter)    | Plasmid                | 3360 U/L (oat spelt xylan) <sup>b</sup>                      | Hydrolysis of sodium hypochlorite-pretreated wheat straw | Morais et al., 2013      |
| <i>Lb. plantarum</i> WCFS1                       | Cel6A endoglucanase plus Xyn11A endoxylanase from <i>T. fusca</i> plus chimeric scaffoldin-AT (synthetic consortium)   | Inducible (sakacin P promoter)    | Plasmid                |  | Hydrolysis of sodium hypochlorite-pretreated wheat straw | Morais et al., 2014      |
| <i>Lb. plantarum</i> WCFS1                       | Chimeric GH5 and GH9 endoglucanases and GH10 and GH11 xylanases from <i>Clostridium papyrosolvens</i> plus chimeric adaptor and anchoring scaffoldins (synthetic | Inducible (sakacin P promoter)    | Plasmid                | 0.2-59.1 nM <sup>b, c</sup>                                  | Hydrolysis of sodium hypochlorite-pretreated wheat straw | Stern et al., 2018       |

|   |   |                              |         |  |  |                              |
|---|---|------------------------------|---------|--|--|------------------------------|
|   | consortium)   |                              |         |  |  |                              |
| <i>Lb. reuteri</i> XC1                      | CelW endoglucanase from <i>Bacillus subtilis</i> WL001 and phyW phytase from <i>Aspergillus fumigatus</i> WL002 (artificial operon) | Constitutive (LdhL promoter) | Plasmid | 960 U/L (CMC) <sup>b</sup>                                     | Hydrolysis of CMC                                | Wang et al., 2014            |
| <i>Lc. lactis</i> HtrA NZ9000               | Fragments of CipA scaffoldin from <i>C. thermocellum</i>  | Inducible (nisA promoter)    | Plasmid | 9 x 10 <sup>3</sup> scaffolds/cell <sup>d</sup>                | Scaffoldins displayed on the cell surface        | Wieckzoreck and Martin, 2010 |
| <i>Lc. lactis</i> IL1403                    | BglA $\beta$ -glucan glucohydrolase and EngD Endoglucanase/Xylanase from <i>Clostridium cellulovorans</i> (artificial operon)       | Constitutive (P32 promoter)  | Plasmid | 1.220 U/L (pNGP) <sup>b</sup> ; 157 U/L (Azo-CMC) <sup>b</sup> | Hydrolysis of CMC; Growth on cellooctaose        | Gandini et al., 2017         |
| <i>Lc. lactis</i> strains IL1403 and MG1363 | Cellulase from <i>Neocallimastix</i> sp.  | Inducible (lacZ promoter)    | Plasmid | 5.9 U (CMC) <sup>b, e</sup>                                    | Hydrolysis of CMC                                | Ozkose et al., 2009          |
| <i>Lc. lactis</i> MG1316                    | Xylanase from <i>Bacillus coagulans</i> ST-6  | Constitutive (P32 promoter)  | Plasmid | ≈87 U/L (xylan) <sup>c</sup>                                   | Hydrolysis of RBB-xylan                          | Raha et al., 2006            |
| <i>Lc. lactis</i> MG1316                    | Egl3 endoglucanase from <i>Trichoderma reesei</i>   | Constitutive (P32 promoter)  | Plasmid | 1118 U/L (CMC) <sup>b</sup>                                    | Improved metabolization of paper and wheat straw | Liu et al., 2017             |

<sup>a</sup>Maximum values reported in each study. Substrates used for determining enzyme activity are indicated in parentheses. Azo-CMC, carboxy methyl cellulose; N3-G5- $\beta$ -CNP, 2-chloro-4-nitrophenyl-6<sup>5</sup>-azido-6<sup>5</sup>-deoxy- $\beta$ -maltopentaoside; PASC, phosphoric acid-swollen cellulose; pNGP, p-nitrophenyl-b-D-glucopyranoside (pNGP); RBB-xylan, remazol brilliant blue xylan .

<sup>b</sup>Enzyme activity/protein quantification measured in extracellular fraction

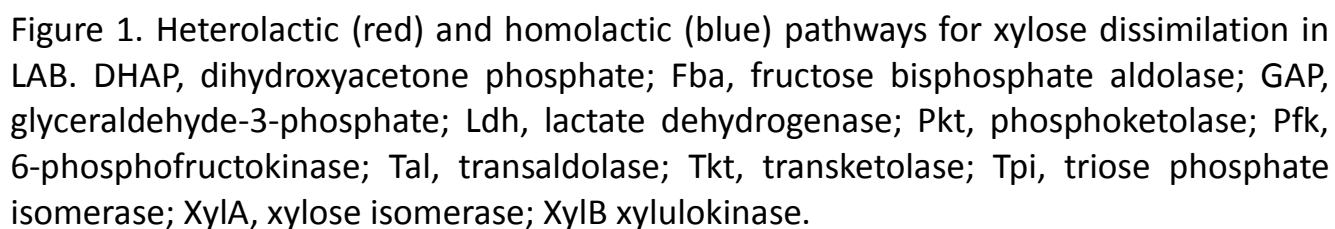
<sup>c</sup>Protein quantification through ELISA-based binding assays on cultures with OD<sub>600nm</sub>=1

<sup>d</sup>Proteins displayed on the cell surface

<sup>e</sup>The volume of extracellular extract used in this study was not reported

Table 3. Recombinant LAB showing improved pentose metabolism. Tkt, transketolase; XylA, xylose isomerase; XylB, xylulose kinase

| Strains   | Heterologous protein(s) expressed  | Transcriptional promoter                                   | Gene cloning strategy              | Improved phenotypic properties of the strain       | References            |
|---|--|--|------------------------------------|--|-----------------------|
| <i>Lb. plantarum</i> NCIMB 8826 ( $\Delta ldh1-xpk1$ )      | Tkt from <i>Lc. lactis</i> IL1403 (replacing endogenous phosphoketolase Xpk1)  | Not indicated  | Chromosome integration             | Almost homolactic (D-LA) fermentation of arabinose | Okano et al. 2009a    |
| <i>Lb. plantarum</i> NCIMB 8826 ( $\Delta ldh1-xpk1-xpk2$ ) | Tkt from <i>Lc. lactis</i> IL1403 (replacing endogenous phosphoketolase Xpk1 and Xpk2); XylA and XylB from <i>Lb. pentosus</i> NRIC 1069   | Not indicated  | Chromosome integration             | Almost homolactic (D-LA) fermentation of xylose    | Okano et al. 2009b    |
| <i>Lc. lactis</i> IL1403 ( $\Delta pkt$ )                   | XylA and XylB from <i>Lc. lactis</i> IO-1 and endogenous tkt replacing endogenous phosphoketolase ( <i>pkt</i> )   | Inducible (xylose) for XylAB. Not indicated for <i>tkt</i> | Plasmid and Chromosome integration | Almost homolactic (L-LA) fermentation of xylose    | Shinkawa et al., 2011 |
| <i>Ped. acidilactici</i> TY112 ( $\Delta ldhD-pkt-ackA2$ )  | Transaldolase, tkt (replacing endogenous phosphoketolase, <i>pkt</i> ), XylA and XylB (replacing endogenous acetate kinase, <i>ackA2</i> ) from <i>Pediococcus acidilactici</i> DSM20284 | Constitutive ( <i>PldhD</i> )                              | Chromosome integration             | Almost homolactic (L-LA) fermentation of xylose    | Qiu et al., 2018      |





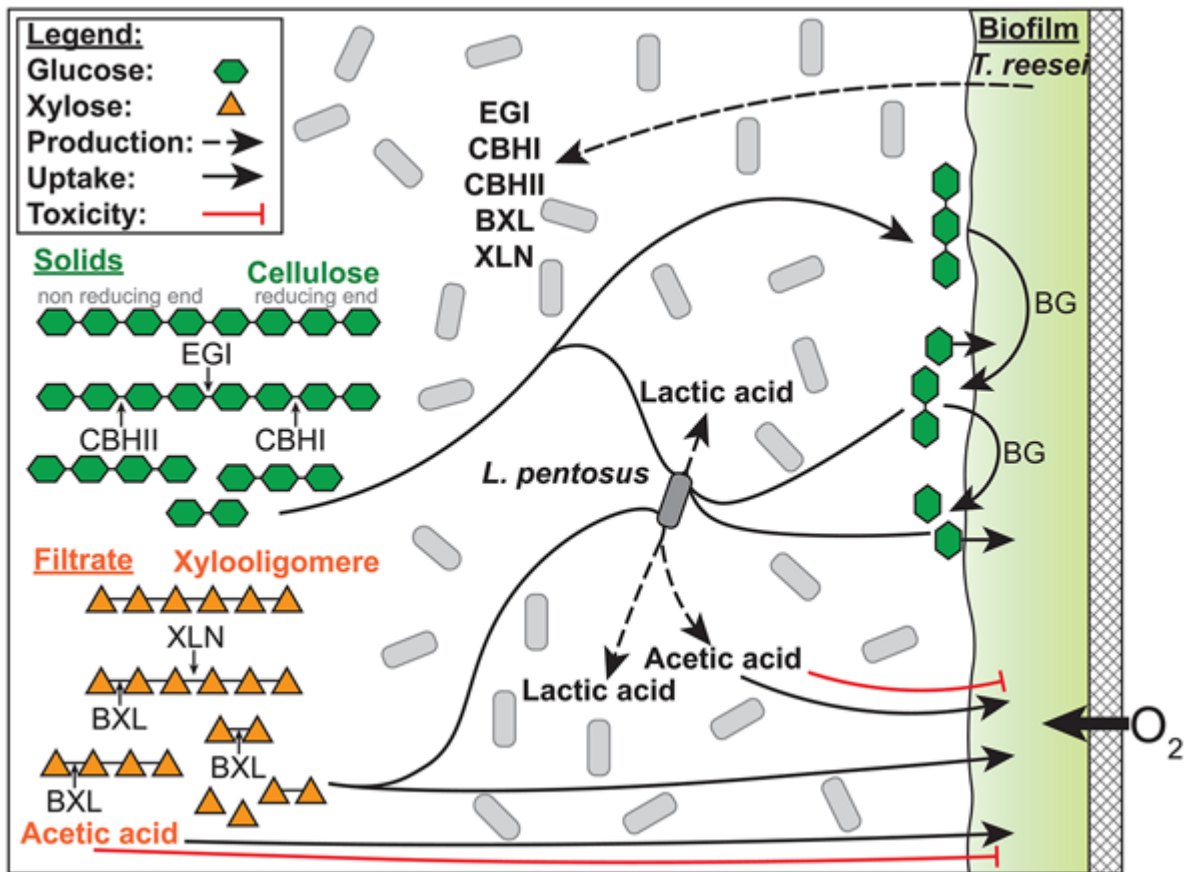


Figure 2. Schematic representation of *T. reesei*/*Lb. pentosus* consortium developed by Shahab et al. (2018). *T. reesei* grows as a biofilm on the surface of an oxygen permeable membrane and secretes cellulases and hemicellulases (EGI: endoglucanase I, CBHI: cellobiohydrolase I, CBHII: cellobiohydrolase II, BXL:  $\beta$ -xylosidase, XLN:  $\beta$ -endoxylanase). Soluble saccharides produced by *T. reesei* enzymes are fermented by *Lb. pentosus* to lactic and acetic acid. Acetic acid can serve as energy source for *T. reesei* (modified from Shahab et al. 2018).

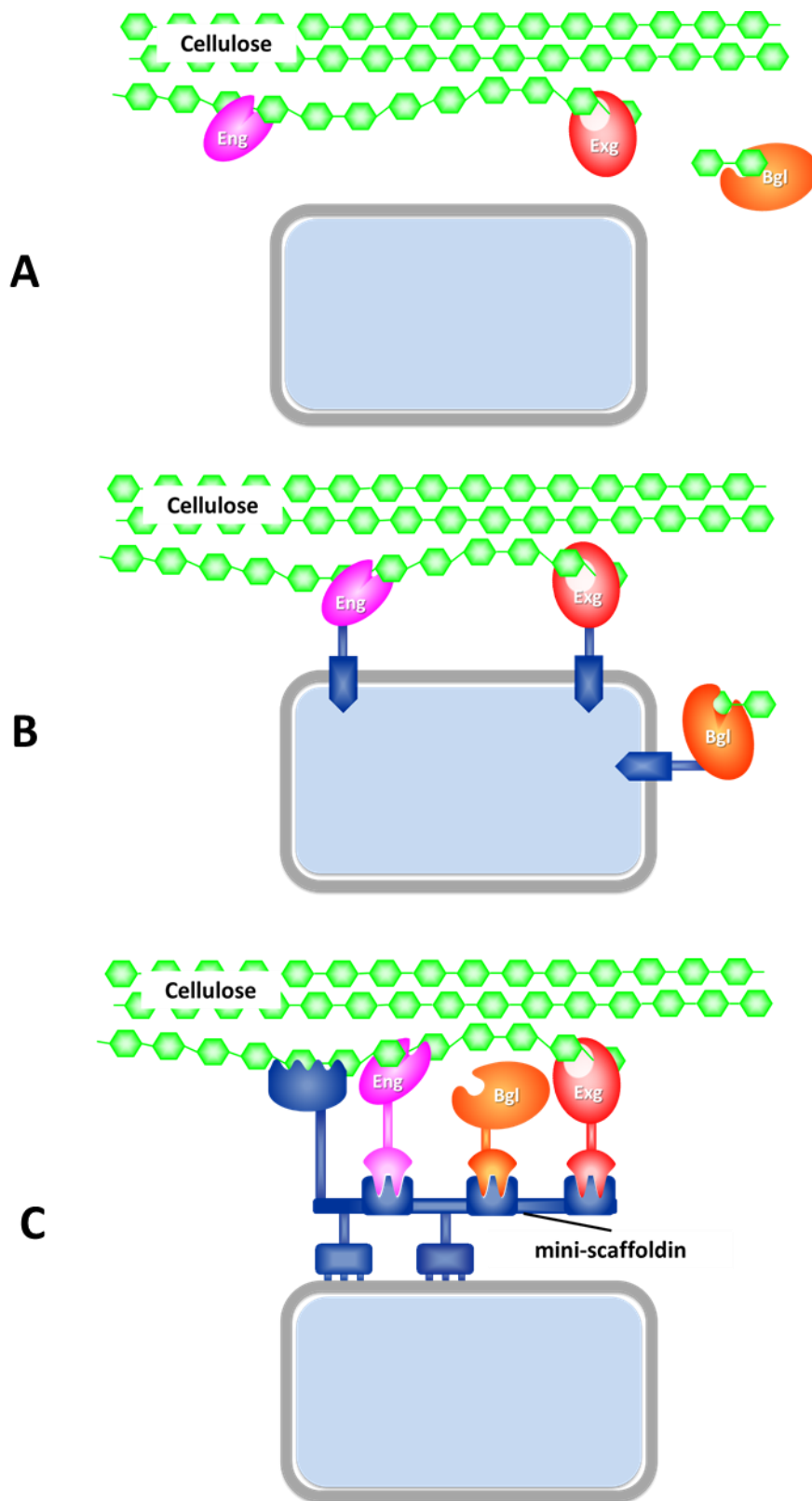


Figure 3. Paradigms for recombinant cellulolytic strategies reported in LAB. Recombinant cells A) secreting minimal non-complexed cellulase system or biosynthesizing B) surface-displayed cellulases or C) surface-displayed designer cellulosomes are depicted. Bgl,  $\beta$ -glucosidase; Eng, endoglucanase; Exg, exoglucanase.